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Gamma Interferon Blocks Gammaherpesvirus Reactivation from Latency in a Cell Type-Specific Manner▽

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Gammaherpesviruses are important pathogens whose lifelong survival in the host depends critically on their capacity to establish and reactivate from latency, processes regulated by both viral genes and the host immune response. Previous work has demonstrated that gamma interferon (IFN-γ) is a key regulator of chronic infection with murine gammaherpesvirus 68 (γHV68), a virus that establishes latent infection in B lymphocytes, macrophages, and dendritic cells. In mice deficient in IFN-γ or the IFN-γ receptor, γHV68 gene expression is altered during chronic infection, and peritoneal cells explanted from these mice reactivate more efficiently ex vivo than cells derived from wild-type mice. Furthermore, treatment with IFN-γ inhibits reactivation of γHV68 from latently infected wild-type peritoneal cells, and depletion of IFN-γ from wild-type mice increases the efficiency of reactivation of explanted peritoneal cells. These profound effects of IFN-γ on chronic γHV68 latency and reactivation raise the question of which cells respond to IFN-γ to control chronic γHV68 infection. Here, we show that IFN-γ inhibited reactivation of peritoneal cells and spleen cells harvested from mice lacking B lymphocytes, but not wild-type spleen cells, suggesting that IFN-γ may inhibit reactivation in a cell type-specific manner. To directly test this hypothesis, we expressed the diphtheria toxin receptor specifically on either B lymphocytes or macrophages and used diphtheria toxin treatment to deplete these specific cells in vivo and in vitro after establishing latency. We demonstrate that macrophages, but not B cells, are responsive to IFN-γ-mediated suppression of γHV68 reactivation. These data indicate that the regulation of gammaherpesvirus latency by IFN-γ is cell type specific and raise the possibility that cell type-specific immune deficiency may alter latency in distinct and important ways.

The human gammaherpesviruses are important pathogens, particularly given their association with lymphomas and lymphoproliferative disease in immunocompromised patients. Given the strict species specificity of Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) and the complexity of clinical studies in infected humans, we have turned to analysis of infection of mice with gammaherpesvirus 68 (γHV68) as a tractable small-animal pathogenesis and immunologic model for understanding the dynamics and genetics of chronic and latent gammaherpesvirus infection. γHV68 was isolated from a bank vole and infects both outbred and laboratory mice. Moreover, γHV68 infection is associated with the development of lymphomas and lymphoproliferative disease in mice (27, 30).

Similar to human gammaherpesviruses, γHV68 establishes both an acute lytic infection and a lifelong latent infection. Human gammaherpesviruses have a propensity to establish latent infection in lymphoid organs, such as spleen and lymph nodes (EBV and KSHV), or in pleural and peritoneal cells (KSHV). Acute γHV68 infection is detectable in multiple organs, while chronic infection is found in the spleen, bone marrow, lung, and peritoneal cells (11, 26, 28, 29, 33, 35, 36), where the virus persists in B cells, macrophages, and dendritic cells (10–12, 29, 35, 36). Control of chronic gammaherpesvirus infection is of particular interest given that reactivation from latency and persistent replication likely contribute to viral spread and disease pathogenesis. Previous work identified gamma interferon (IFN-γ) as a key regulator of γHV68 reactivation from latency (25, 31, 34). Mice genetically deficient in IFN-γ or IFN-γ receptor (IFN-γR) are unable to control chronic γHV68 infection, as evidenced by increased efficiency of reactivation from latency and persistent low-level productive infection (25, 31). IFN-$\gamma^{-/-}$ and IFN-$\gamma R^{-/-}$ mice ultimately develop a large-vessel vasculitis which is dependent on viral replication in the immunoprivileged media of the great elastic arteries (8, 34). Moreover, the addition of IFN-γ to wild-type latently infected peritoneal cells ex vivo significantly reduces the frequency of reactivation events (25).

While it is clear that IFN-γ signaling is essential for the control of chronic gammaherpesvirus infection, the mechanism by which IFN-γ exerts its effects and the relevant cell types targeted by IFN-γ are unknown. Based on our previous studies, it is likely that IFN-γ suppresses reactivation from latency by signaling directly to infected cells (25). Given that B cells and macrophages constitute the predominant cell types that harbor latent virus in splenocytes and peritoneal cells, we investigated whether one or both of these cell types represent the relevant targets of IFN-γ for suppression of γHV68 reactivation.

IFN-γ inhibits reactivation of γHV68 in peritoneal cells, but not splenocytes. As previously reported (25), IFN-γ treatment suppresses the frequency of γHV68 reactivation from perito-

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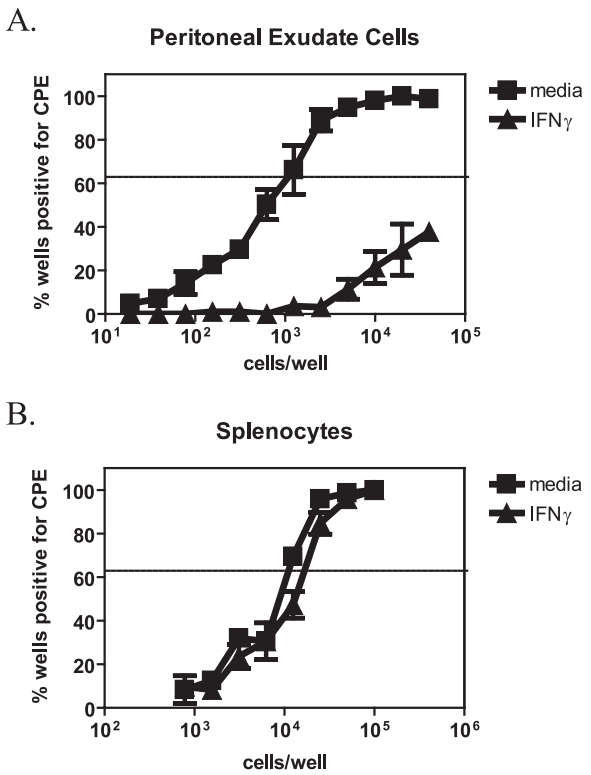

FIG. 1. IFN-γ inhibits reactivation of γHV68 in peritoneal cells, but not splenocytes, isolated from latently infected wild-type mice. (A) Ex vivo reactivation assay demonstrating that γHV68 latently infected peritoneal cells treated with 100 U/ml of IFN-γ reactivate at a lower frequency than peritoneal cells treated with medium only. (B) In contrast to peritoneal cells, IFN-γ treatment of splenocytes did not alter the frequency of γHV68 reactivation. Reactivation from latency was assayed by plating limiting dilutions of cells onto permissive MEF monolayers and scoring for CPE as a result of infectious virus 3 weeks later. Serial twofold dilutions of cells (24 wells/dilution) were plated onto an indicator monolayer of IFN-αβγR$^{-/-}$ MEFs in 96-well tissue culture plates. To distinguish between reactivation from latency and preformed infectious virus present in these cells, we plated parallel cell samples after mechanical disruption.

neal cells harvested from latently infected mice in an ex vivo reactivation assay (Fig. 1A). Peritoneal cells harvested from animals 16 days postinfection plated in medium alone reactivated with an approximate frequency of 1/1,000, while cells treated with 100 units/ml of IFN-γ reactivated at a substantially lower frequency, conservatively 1/40,000. In contrast, splenocytes harvested from mice 16 days postinfection reactivate γHV68 at a frequency of approximately 1/10,000 when plated in either medium only or treated with 100 units/ml of IFN-γ (Fig. 1B). Viral reactivation from latency was solely responsible for the observed cytopathic events, as mechanical disruption of parallel samples resulted in no cytopathic effect (CPE) (data not shown).

Previous work from our laboratory supports the notion that IFN-γ has site-specific effects on γHV68 reactivation. Specifically, in IFN-γ$^{-/-}$ deficient mice, reactivation from latency is dramatically increased in peritoneal cells, while viral reactivation from splenocytes is slightly decreased compared to wild-type cells (31).

Together these data demonstrate that IFN-γ exerts its effects selectively, altering reactivation from peritoneal cells but not splenocytes. Given that the predominant latent reservoir of γHV68 in peritoneal cells is the macrophage, and in the spleen it is the B cell (36, 37), we hypothesized that the difference in the ability of IFN-γ to suppress reactivation in cells derived from these two anatomic sites reflects a cell type-specific effect of IFN-γ on γHV68 reactivation.

IFN-γ inhibits reactivation of γHV68 in peritoneal cells and splenocytes from B-cell-deficient mice. One prediction of this model was that B cells, the predominant cell carrying latent γHV68 in the spleen, would be unresponsive to the effects of IFN-γ. To investigate whether IFN-γ can suppress γHV68 reactivation from latency in splenocytes lacking B cells, we examined the effect of IFN-γ on γHV68 reactivation in splenocytes from B-cell-deficient (μMT) animals. These mice are deficient in mature B cells due to a homozygous mutation in the μ heavy-chain transmembrane exon (17). Previous work has demonstrated that γHV68 latency is established in both peritoneal cells and splenocytes in the absence of mature B cells in these mice (19, 33). Despite the fact that B cells are the predominant cell type harboring γHV68 latency in the spleen in wild-type mice (37), splenocytes from B-cell-deficient mice reactivate latent γHV68 virus (19, 33).

Consistent with prior investigations, γHV68 reactivated from μMT peritoneal cells 16 days postinfection with a frequency of 1/127 (Fig. 2A). IFN-γ treatment decreased the frequency of cells reactivating γHV68 to 1/3,232, an approximate 25-fold decrease. Strikingly, the addition of IFN-γ to splenocytes lacking B cells also significantly reduced the frequency of cells reactivating γHV68 (Fig. 2B). Approximately 1/17,000 splenocytes from B-cell-deficient mice reactivated from latency when plated in medium only, and addition of 100 units/ml of IFN-γ reduced the frequency of reactivation events to negligible levels. These results demonstrate that IFN-γ can suppress reactivation of γHV68 in both the peritoneal cell and splenocyte populations harvested from B-cell-deficient animals. This suggests that IFN-γ acts on non-B cells to inhibit reactivation.

Strategy for selective depletion of B cells and macrophages in vivo. In order to further investigate the cell type-specific effects of IFN-γ on γHV68 reactivation, we employed a genetic strategy to deplete specific cells in a temporally controlled fashion. To accomplish this, we bred mice carrying both a Cre recombinase-inducible simian diphtheria toxin receptor (DTR) in the *ROSA26* locus (iDTR mice [4]) and Cre recombinase expressed specifically in either B cells (CD19-Cre) or macrophages (lysozyme-Cre). Injection of diphtheria toxin into iDTR/Cre mice allows the specific depletion of DTR-expressing macrophages or B cells in a temporally controlled manner. This system has several advantages. Most importantly, it allows the depletion of macrophages or B cells after the establishment of latency. This enables us to analyze immune control of latency in mice with normal immune systems.

Selective depletion of B cells after the establishment of γHV68 latency in vivo. To generate mice in which B cells can be acutely depleted, we bred iDTR mice with mice expressing Cre from the B-cell-specific CD19 promoter (CD19-Cre) (22) to generate iDTR/CD19-Cre mice. CD19 is expressed throughout B-cell development (18, 38).

iDTR/CD19-Cre mice were infected with γHV68. Sixteen

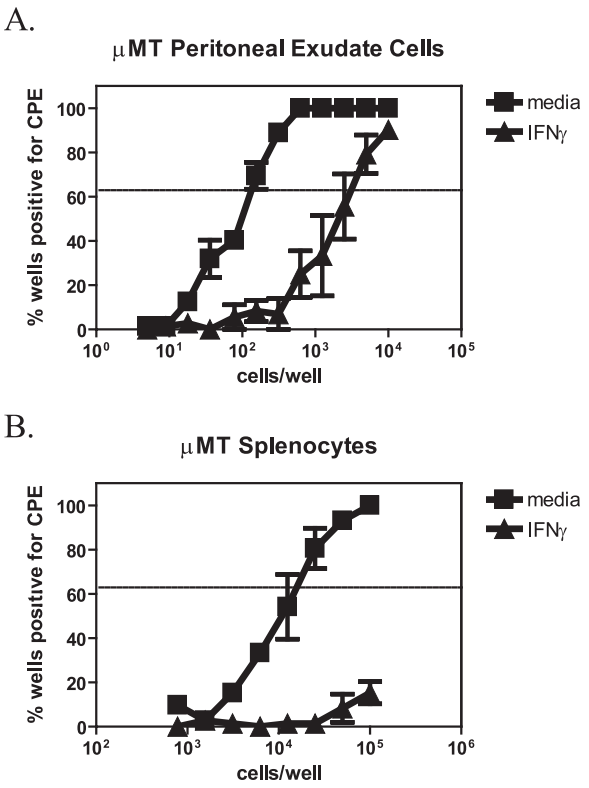

FIG. 2. IFN-γ inhibits reactivation of γHV68 in peritoneal cells and splenocytes isolated from latently infected B-cell-deficient mice. (A) Ex vivo reactivation assay demonstrating that compared to treatment with medium only, IFN-γ (100 U/ml) treatment of latently infected peritoneal cells isolated from B-cell-deficient mice (μMT) reduces the frequency of γHV68 reactivation. (B) In contrast to splenocytes isolated from wild-type mice, IFN-γ treatment of latently infected B-cell-deficient splenocytes reduces the frequency of γHV68 reactivation compared to treatment with medium only.

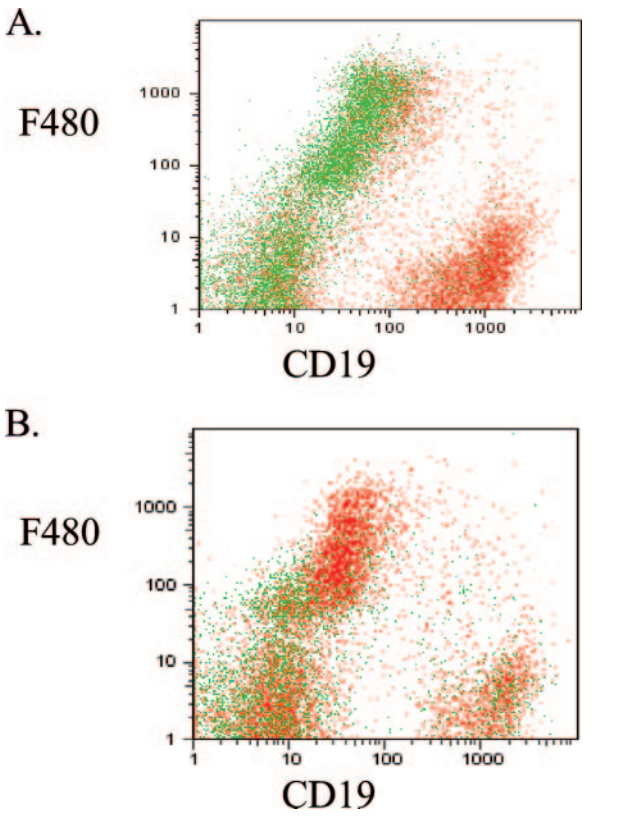

FIG. 3. Depletion of B cells and macrophages from the peritoneal cavities of iDTR/CD19-Cre and iDTR/Lyz-Cre mice treated with diphtheria toxin. Representative fluorescence-activated cell sorting analysis of peritoneal cells isolated from DT-treated (green) and mock-treated (red) iDTR/CD19-Cre (A) and iDTR/Lyz-Cre (B) latently infected animals. (A) Intraperitoneal injection of 100 ng of DT daily for 7 days led to significant depletion of CD19-positive cells from iDTR/CD19-Cre mice. (B) Intraperitoneal injection of 100 ng DT daily for 7 days resulted in a significant reduction of F480^{high} cells from iDTR/Lyz-Cre mice. F480 staining for macrophages is shown on the *y* axis, and CD19 staining for B cells is shown on the *x* axis.

days later, mice were injected with either 100 ng of diphtheria toxin (DT) or bovine serum albumin (BSA) (control) once a day for 7 days. Peritoneal cells were then harvested and analyzed by flow cytometry for macrophages (F480), B cells (CD19), T cells (CD4 and CD8), and dendritic cells (Cd11c, analyzed in the spleen only) to ascertain the extent and specificity of depletion. DT administration did not affect dendritic cells or CD4 or CD8 T cells (data not shown). In contrast, significant depletion of B cells from the peritoneal cell population (94% depletion) was demonstrated in the iDTR/CD19-Cre mice treated with DT compared to mock-treated animals (Fig. 3A).

Depletion of B cells from peritoneal cells does not affect the frequency of cells reactivating γHV68. We first determined whether B cells are required for reactivation or IFN-γ-mediated suppression of γHV68 latency from peritoneal cells. Cells harvested from iDTR/CD19-Cre mice treated with DT or BSA after the establishment of latency were analyzed in explant reactivation assays. Peritoneal cells from mock-treated animals reactivated at a frequency of approximately 1/14,500 (Fig. 4A). Cells harvested from iDTR/CD19-Cre mice treated with DT demonstrated no differences in the frequency of reactivation compared to cells from mock-treated animals despite significant depletion of B cells. These results indicate that, consistent with prior studies using flow cytometry to sort cell populations (36), B cells are not the primary peritoneal cells responsible for γHV68 reactivation. IFN-γ significantly reduced the frequency of reactivation in peritoneal cells harvested from either DT- or mock-treated iDTR/CD19-Cre animals, indicating that peritoneal cell types other than B cells, likely macrophages, are the target for IFN-γ-mediated suppression of reactivation.

Selective depletion of macrophages expressing high levels of F480 (F480^{high} macrophages) in the peritoneal cell population after the establishment of γHV68 latency in vivo. To investigate the importance of macrophages in IFN-γ-mediated suppression of γHV68 reactivation after the establishment of latency, we bred iDTR mice to mice expressing Cre from the endogenous lysozyme M locus (Lyz-Cre) (6). Lysozyme M expression is restricted to myelomonocytic cells, specifically macrophages and neutrophil granulocytes (3, 7, 9). iDTR/Lyz-Cre mice were infected with γHV68, and after the establishment of latency 16 days postinfection, the mice were injected with DT or BSA once a day for 7 days. Peritoneal cells were then harvested and subjected to fluorescence-activated cell

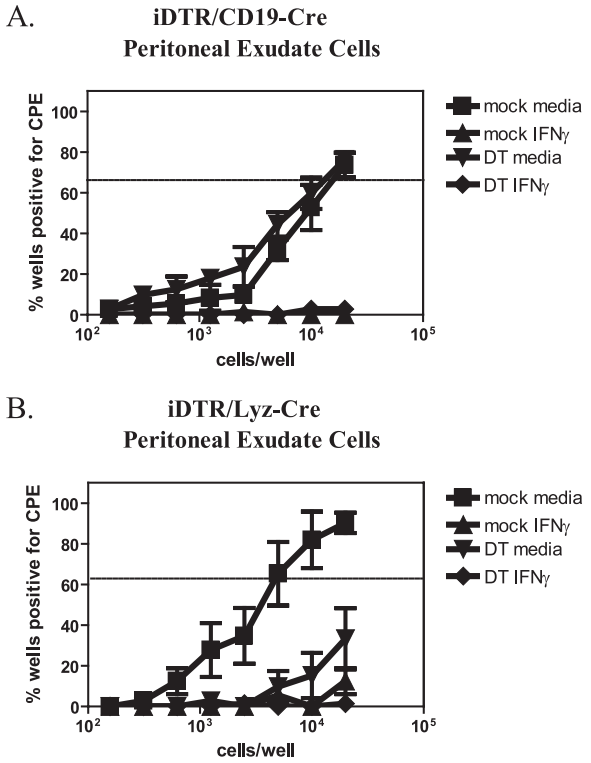

FIG. 4. Macrophages but not B cells are responsible for γHV68 reactivation from latently infected peritoneal cells. (A) Compared to controls, depletion of B cells from the peritoneal cavities of latently infected mice (iDTR/CD19-Cre) does not affect the frequency of peritoneal cells reactivating γHV68. Moreover, depletion of B cells did not alter the ability of IFN-γ to suppress viral reactivation. (B) Depletion of macrophages from the peritoneal cavities of latently infected mice (iDTR/Lyz-Cre) significantly reduced the frequency of peritoneal cells reactivating γHV68. IFN-γ treatment reduced the frequency of reactivation from both mock-treated and macrophage-depleted peritoneal cells.

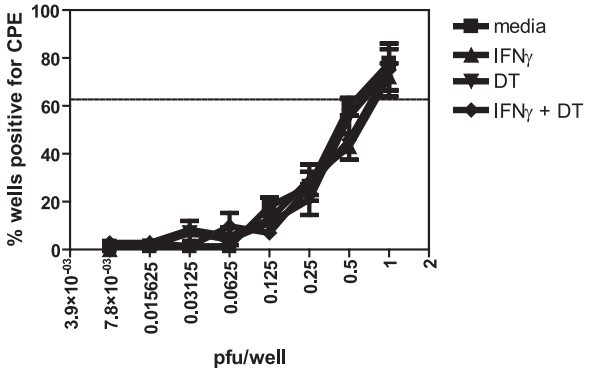

FIG. 5. DT and IFN-γ treatment of MEFs do not affect the ability of γHV68 to induce CPE. γHV68 was added in a limiting dilution fashion to indicator MEF monolayers treated with DT and/or IFN-γ. The ability of γHV68 to promote CPE in the presence of DT and/or IFN-γ was unaffected after 10 days.

sorting analysis. No differences in the dendritic, CD4, or CD8 T-cell populations in harvested peritoneal cells were observed between DT- and mock-treated animals (data not shown). DT treatment resulted in about a 50% depletion of macrophages expressing F480 (Fig. 3B). The F480 monoclonal antibody recognizes a macrophage-restricted 160-kDa glycoprotein (1, 13–16, 20). Of the F480-positive peritoneal cells harvested from the DT-treated mice, the mean fluorescence intensity of F480 staining was markedly decreased compared to that of peritoneal cells harvested from the mock-treated animals. Thus, DT selectively deleted F480^{high} macrophages, leaving a macrophage population expressing low levels of F480. Additionally, analysis of forward and side scatter revealed that DT treatment resulted in loss of a significant proportion of large and granular peritoneal cells (data not shown).

Depletion of F480^{high} macrophages from peritoneal cells significantly reduces the frequency of cells reactivating γHV68. To determine whether macrophages play a cell type-specific role in IFN-γ-mediated suppression of γHV68 latency, we next analyzed peritoneal cells harvested from iDTR/Lyz-Cre mice treated with once-daily DT or BSA injections for 7 days after the establishment of latency in the ex vivo reactivation assay. Peritoneal cells harvested from the mock-treated animals reactivated at a frequency of 1/5,250 (Fig. 4B). Cells harvested from the DT-treated iDTR/Lyz-Cre mice reactivated at a low frequency, conservatively 1/20,000, a statistically significant decrease in the frequency of reactivation compared to cells from the mock-treated animals ($P = 0.01$). These data demonstrate that cells expressing Cre from the lysozyme M promoter are responsible for the majority of the reactivation events from the peritoneal cell population.

As expected, IFN-γ treatment of cells from mock-treated animals significantly reduced the frequency of reactivation, conservatively 1/20,000 (Fig. 4B). IFN-γ treatment of peritoneal cells harvested from DT-treated iDTR/Lyz-Cre animals also reduced the frequency of cells reactivating γHV68, conservatively 1/20,000. These data were consistent with the interpretation that IFN-γ inhibits reactivation of γHV68 from macrophages, but interpretation of this experiment was complicated by the presence of a residual F480-positive population in DT-treated iDTR/Lyz-Cre mice.

Macrophages but not B cells are responsible for reactivation of latent γHV68 from peritoneal cells. To further address the hypothesis that macrophages are the target of IFN-γ-mediated suppression of γHV68 reactivation, we used treatment of explanted cells with DT. We harvested latently infected cells and treated them with either BSA or 100 ng/ml of DT in vitro upon explant in the limiting dilution reactivation assay.

First, to confirm the validity of explant reactivation assays in the presence of DT, we examined whether addition of DT affected our capacity to detect γHV68 CPE on the indicator monolayers of murine embryonic fibroblasts used to detect γHV68 reactivation. γHV68 was diluted onto indicator layers of mouse embryonic fibroblasts (MEFs) in the presence or absence of DT (100 ng/ml), IFN-γ (100 units/ml), or both DT and IFN-γ together (Fig. 5). None of these treatments altered detection of γHV68 infection. As we utilize IFN-αβγR$^{-/-}$ MEFs in this assay, we did not expect IFN-γ to have any effect of reactivation, confirming the results of earlier studies (25).

iDTR/Lyz-Cre and iDTR/CD19-Cre animals were infected with γHV68, and peritoneal cells were harvested 16 days later after the establishment of latency. These cells were placed into

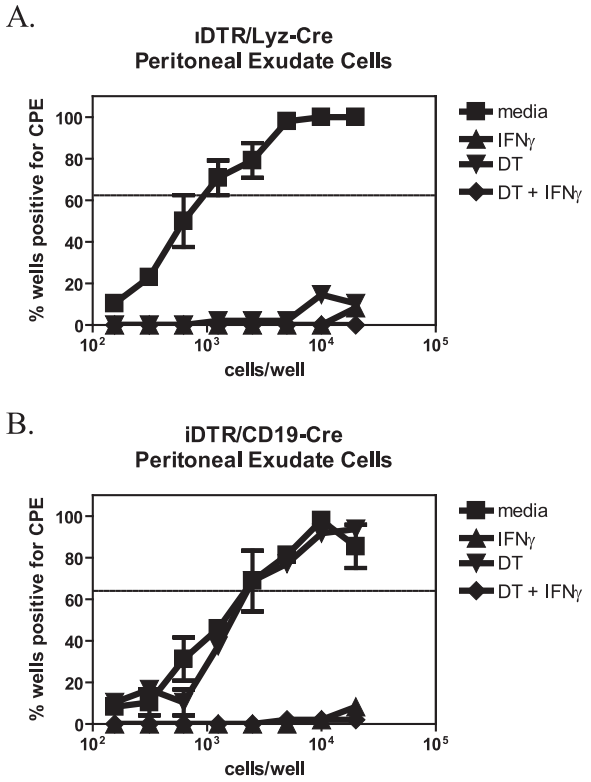

FIG. 6. Ex vivo DT treatment demonstrates that macrophages but not B cells are the predominant latently infected cell responsible for γHV68 reactivation from the peritoneal cell population. (A) Ex vivo depletion of macrophages from peritoneal cells of latently infected mice (iDTR/Lyz-Cre) significantly reduced the frequency of peritoneal cells reactivating γHV68. IFN-γ treatment reduced the frequency of reactivation of control depleted peritoneal cells. (B) Compared to controls, ex vivo depletion of B cells from peritoneal cells isolated from latently infected mice (iDTR/CD19-Cre) does not affect the frequency of peritoneal cells reactivating γHV68. Moreover, depletion of B cells did not alter the ability of IFN-γ to suppress viral reactivation.

explant cultures in limiting dilutions in either medium alone or medium supplemented with DT (100 ng/ml), IFN-γ (100 units/ml), or both DT and IFN-γ together. Cells plated in medium alone reactivated with a frequency of approximately 1/1,000, as expected. The addition of DT to latently infected peritoneal cells harvested from iDTR/Lyz-Cre animals almost completely abolished reactivation (Fig. 6A). The frequency of cells reactivating γHV68 in the presence of IFN-γ-containing medium was significantly decreased (but not eliminated). Importantly, no reactivation was observed in any cultures treated with both DT- and IFN-γ-containing medium. This result likely reflects the ability of IFN-γ to suppress reactivation in the small population of cells capable of reactivating γHV68 following DT treatment. These data demonstrate that ex vivo depletion of Lyz-Cre-expressing cells reduces the frequency of γHV68 reactivation, allowing us to conclude that macrophages are the predominant cell type capable of reactivating virus.

Similarly, ex vivo reactivation assays utilizing peritoneal cells from iDTR/CD19-Cre mice treated with DT and/or IFN-γ produced findings consistent with the results of our in vivo experiments. Addition of DT to peritoneal cells from iDTR/CD19-Cre mice in vitro did not alter the frequency of cells reactivating γHV68 (approximately 1/2,150 and 1/2,880 in the cultures treated with medium alone and supplemented with DT, respectively; $P > 0.05$; Fig. 6B). Thus, B cells are not the predominant cell type responsible for γHV68 reactivation from latently infected peritoneal cells. As expected, the addition of IFN-γ alone or in the presence of DT in these cultures reduced the frequency of reactivation events significantly. These data combined with data from the above experiments utilizing iDTR/Lyz-Cre mice indicate that macrophages but not B cells are the targets for IFN-γ regulation of reactivation.

Implications for cell type-specific γHV68 latency. γHV68 latency is established and maintained in various cellular subsets in distinct anatomical locations (11, 26, 28, 29, 33, 35, 36). For example, in peripheral blood, the major viral reservoir is isotype-switched B cells, while chronic infection in the lung is established in a variety of cells, including epithelial cells as well as macrophages, B cells, and dendritic cells (10–12, 29, 35, 36). Given these differences in cell reservoirs between different anatomical locations, it seems likely that γHV68 establishes and maintains latency through distinct viral programs in different cell types. This idea is supported by the characterization of different latency programs for EBV each with distinct patterns of viral transcription (reviewed in reference 23).

The program of viral gene expression in distinct reservoirs of γHV68 latency is not well characterized. Prior studies have uncovered that viral latency is established in distinct cell types in various anatomical locations. For instance, in the lung, latent γHV68 infection can be detected in B cells, whereas in the spleen, latent γHV68 infection is established in macrophages, dendritic cells, and B cells (11, 26, 28, 29, 33). Despite these and other studies describing the locations and cell types harboring latent infection, little is known regarding potential differences in the molecular mechanisms controlling establishment and reactivation from viral latency in these distinct cell types. Studies comparing viral gene expression from latently infected mesenteric lymph nodes and splenocytes demonstrate distinct expression profiles in these cell types, including M11 and K3 (24). Moreover, previous work from our laboratory has suggested that different viral programs may operate in peritoneal cells versus splenocytes. Comparisons of viral gene expression from latently infected peritoneal exudate cells and splenocytes reveal significant differences in the expression levels of several genes (2, 25, 31, 34). Furthermore, using nested reverse transcription-PCR to detect transcription in genomic areas corresponding to known latency-associated genes in other gammaherpesviruses, different profiles of viral gene expression in latently infected peritoneal cells and splenocytes were detected (32). Interestingly, the characterization of a spontaneous 9.5-kb deletion mutation in γHV68 revealed that there are different genetic requirements for the establishment, maintenance, and reactivation from latency in spleen cells compared to peritoneal cells (5). The possibility that γHV68 latency operates via distinct viral programs in different anatomical locations means that the immune system may face different challenges in controlling chronic infection in different sites. In this paper we show that IFN-γ has distinct effects on macrophages versus B cells, consistent with the concept that immune cytokines have cell type-specific effects on viral latency in vivo.

Mechanisms of cell type-specific effects of IFN-γ on γHV68 latency. One explanation for the cell type-specific effects of IFN-γ lies at the level of cell type-specific responses to IFN-γ. For instance, IFN-γ may induce an antiviral state in the macrophage via cell type-specific mechanisms that are not induced or effective in a B cell. For control of cytomegalovirus infection in macrophages, the effects of IFN-γ differ dramatically in macrophages and fibroblasts, and this correlates with distinctly different patterns of IFN-γ-mediated gene transcription (21). This type of mechanism would predict that the IFN-γ response of primary macrophages and B cells differs with respect to the molecules responsible for IFN-γ effects on reactivation from latency. Addressing this fundamental question will require identification of the molecular mechanisms of IFN-γ inhibition of reactivation and then comparing different cell types for those mechanisms.

Since IFN-γ signaling is unable to suppress reactivation from latency in B cells, it is likely that a separate component of the immune system regulates viral reactivation in the B-cell reservoir. Prior work has implicated other site-specific immune system components, such as perforin, as necessary for the control of chronic infection (31). Perforin acts primarily in the spleen to control the number of latently infected cells (presumably B cells). Another candidate is IFN-α/β, as deletion of the IFN-α/βR chain 1 leads to increased reactivation and altered viral gene expression in the spleen (2). In addition to perforin and the type I interferons, it is possible that a number of other not yet identified factors control latent infection in the B-cell reservoir.

The existence of distinct viral latency programs and the ability of the immune system to control chronic gammaherpesvirus infection in a cell-specific manner have significant implications for our understanding of the mechanisms responsible for control of gammaherpesvirus latency and likely disease pathogenesis. Defining the different γHV68 gene programs operating in cell type-specific manners will allow the identification of cell type-specific mechanisms of immune control of chronic γ-herpesvirus infection.

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